

Detection of *Salmonella typhi* in the Blood of Patients with Typhoid Fever by Polymerase Chain Reaction

JAE-HOON SONG,^{1*} HELEN CHO,² MEE YEON PARK,² DOE SUN NA,³ HEE BOM MOON,¹
AND CHIK HYUN PAI⁴

Departments of Medicine,¹ Biochemistry,³ and Clinical Pathology,⁴ Asan Medical Center, College of Medicine, University of Ulsan, and Asan Institute for Life Sciences,² Seoul, Korea

Received 23 November 1992/Accepted 26 February 1993

A polymerase chain reaction (PCR)-based test was developed for the detection of *Salmonella typhi* in the blood specimens from patients with typhoid fever. Two pairs of oligonucleotide primers were designed to amplify a 343-bp fragment of the flagellin gene of *S. typhi*. Amplified products were analyzed by agarose gel electrophoresis and Southern blot hybridization by using a ³²P-labeled 40-base probe internal to the amplified DNA. The nested PCR with two pairs of primers could detect 10 organisms of *S. typhi* as determined by serial dilutions of DNA from *S. typhi*. The peripheral mononuclear cells from 11 of 12 patients with typhoid fever confirmed by blood culture were positive for DNA fragment of the flagellin gene of *S. typhi*, whereas 10 blood specimens of patients with other febrile diseases were negative. With the nested PCR, *S. typhi* DNAs were detected from blood specimens of four patients with suspected typhoid fever on the basis of clinical features but with negative cultures. We suggest that the PCR technique could be used as a novel diagnostic method of typhoid fever, particularly in culture-negative cases.

Typhoid fever caused by *Salmonella typhi* remains an important public health problem in many parts of the world. Rapid and sensitive laboratory methods for diagnosis of typhoid fever are essential for prompt and effective therapy. Although several serological assays for detecting *S. typhi* antigens or antibodies have been used for their rapidity and simplicity, no nonculture tests for typhoid fever have repeatedly been shown to be highly sensitive and specific (1). The classical and the most commonly used serological method, the Widal test, is particularly unreliable with the single titers in endemic areas (8, 14). Confirmation of typhoid fever requires the identification of *S. typhi* in clinical specimens. *S. typhi* can be isolated from more than 90% of patients with typhoid fever if blood, stool, rose spots, and bone marrow aspirates are all cultured (5). If the culture of the mononuclear cell-platelet layer of blood is combined with cultures of bone marrow aspirate and rectal swab, the positive rate of detection can increase up to 100% (11). Since it is often difficult to obtain bone marrow aspirates in many endemic areas, only blood specimens are cultured in most cases.

Blood culture, however, can detect only 45 to 70% of patients with typhoid fever, depending on the amount of blood sampled, the bacteremic level of *S. typhi*, the type of culture medium used, and the length of incubation period (6, 7). In Korea, where typhoid fever is still common, diagnosis of many suspected cases on the basis of clinical findings cannot be confirmed because of negative cultures. The clinical usefulness of the culture method is further restricted because it takes at least 2 days until the identification of the organism. The development of a rapid and sensitive diagnostic method of typhoid fever, therefore, has a practical importance in endemic areas.

Previously, a DNA probe specific to the Vi antigen of *S. typhi* had been used to detect the organism in the blood of patients with typhoid fever (9, 10, 12). This novel hybridization method, however, required concentration of bacteria

from the blood samples and amplification of total bacterial DNA by overnight incubation of the bacteria on nylon filters to increase the sensitivity of the probe. This process of concentration was inevitable, because patients with typhoid fever usually have less than 15 *S. typhi* cells per ml of blood, and the probe cannot detect fewer than 500 bacteria. The problem of sensitivity of DNA probes could be circumvented by polymerase chain reaction (PCR), which can detect very small amounts of DNA by enzymatic amplification. PCR with the sequences of Vi antigen is not feasible, because the nucleotide sequence of this antigen has not been fully investigated. We report here the development of a PCR-based assay which can detect *S. typhi* DNA by amplification of the flagellin gene of *S. typhi* in the blood of typhoid patients.

MATERIALS AND METHODS

Bacterial strains. Ten *Salmonella* strains, including two strains of *S. typhi*, seven non-*Salmonella* gram-negative organisms, *Listeria monocytogenes*, and *Legionella pneumophila* were studied for a test of specificity of the PCR (Table 1). Nine *Salmonella* strains were obtained from the American Type Culture Collection (ATCC), and one *S. typhi* strain was isolated from a patient with typhoid fever admitted to the Asan Medical Center. All other organisms were blood-borne pathogens isolated at the Asan Medical Center, which were identified by the Automicrobic System (Vitek Inc., Hazelwood, Mo.). *S. typhi* ATCC 19430 was used as a positive reference.

Blood specimens. Blood specimens (3 ml) were obtained before antibiotic therapy from 12 patients with typhoid fever who were admitted to the Asan Medical Center consecutively during the period from December 1990 to March 1991. All cases were confirmed by blood culture. Blood specimens were also collected from 10 control patients with other febrile diseases which could mimic typhoid fever in terms of clinical manifestations. The diagnosis of these control patients were *Escherichia coli* bacteremia (2 patients), *Kleb-*

* Corresponding author.

TABLE 1. Characteristics of *Salmonella* strains and other gram-negative organisms used in this study

Species	Flagellar antigen(s)	Source	Lane no. (Fig. 1)
<i>Salmonellae</i>			
<i>S. typhi</i>	d:	Asan Medical Center	1
<i>S. typhi</i>	d:	ATCC 19430	2
<i>S. paratyphi</i>	a:	ATCC 11511	3
<i>S. schottmuelleri</i>	b:1,2	ATCC 10719	4
<i>S. hirschfeldii</i>	c:1,4,5	ATCC 13428	5
<i>S. thompson</i>	k:1,3,4,5	ATCC 10256	6
<i>S. typhimurium</i>	i:1,2,3	ATCC 13311	7
<i>S. choleraesuis</i>	c:1,3,4,5	ATCC 6958	8
<i>S. enteritidis</i>	gom:	ATCC 13076	9
<i>S. muenchen</i>	d:1,2	ATCC 8388	10
<i>Escherichia coli</i>		Asan Medical Center	12
<i>Citrobacter freundii</i>		Asan Medical Center	13
<i>Klebsiella pneumoniae</i>		Asan Medical Center	14
<i>Enterobacter cloacae</i>		Asan Medical Center	15
<i>Serratia marcescens</i>		Asan Medical Center	16
<i>Proteus mirabilis</i>		Asan Medical Center	17
<i>Pseudomonas aeruginosa</i>		Asan Medical Center	18
<i>Listeria monocytogenes</i>		Asan Medical Center	19
<i>Legionella pneumophila</i>		Asan Medical Center	20

siella bacteremia (2 patients), liver abscess (1 patient), viral meningitis (1 patient), murine typhus (1 patient), and viral infections (3 patients). Blood specimens were also obtained from four patients with suspected typhoid fever on the basis of clinical findings, such as fever, headache, relative bradycardia, rose spots, hepatic dysfunction, leukopenia, and high Widal titer, but with negative cultures for *S. typhi*. The blood specimen from a normal subject was obtained to be used as a negative control. All blood specimens collected in tubes containing citrate were stored at 4°C until processing. The duration of storage was several hours to 1 month.

PCR primers and probes. From the sequence of the flagellin gene of *S. typhi* (4, 13), two pairs of oligonucleotide primers, of which one is nested in the other, were synthesized by use of a DNA synthesizer (model 391; Applied Biosystems, Foster City, Calif.): ST 1 (5'-ACT GCT AAA ACC ACT ACT-3'), ST 2 (5'-TTA ACG CAG TAA AGA GAG-3'), ST 3 (5'-AGA TGG TAC TGG CGT TGC TC-3'), and ST 4 (5'-TGG AGA CTT CGG TCG CGT AG-3'). Oligonucleotides ST 1 and ST 2, which were used in the first round of the PCR to amplify a 458-bp fragment, correspond to nucleotides 1072 to 1089 and 1513 to 1530, respectively, in the flagellin gene of *S. typhi*. Oligonucleotides ST 3 and ST 4, which were used in the nested PCR on the amplified products from the first PCR to amplify a 343-bp fragment, correspond to nucleotides 1092 to 1111 and 1416 to 1435, respectively. We used one additional oligonucleotide internal to the amplified DNA for Southern blot hybridization which correspond to nucleotides 1136 to 1175 (5'-GCG CAA ATG GTA AAT CTG AAG TTG TTA CTG CTA CCG TAG G-3').

Preparation of DNA from bacteria and blood specimens for PCR. Chromosomal DNAs of *Salmonella* strains and other organisms were extracted as previously described (3). After centrifugation at 3,000 × *g* of 0.5 ml of an overnight Luria broth culture, the resulting pellet was resuspended in 75 µl of 50 mM Tris-HCl buffer (pH 8.0) containing 0.9% glucose, 250 mM EDTA, and 140 µg of lysozyme. The reactants were incubated at 37°C for 30 min. Next, 300 µl of 50 mM NaCl containing 1% sodium dodecyl sulfate (SDS) and 800 µg of proteinase K were added, and the incubation was continued

for an additional 120 min. The procedure was followed by phenol-chloroform-isoamylalcohol (25:24:1) extraction. The DNA was precipitated by the addition of absolute ethanol and harvested by centrifugation. The mononuclear cells were obtained from whole blood specimens after density gradient centrifugation with Ficoll-Hypaque (Histopaque; Sigma Chemical Co., St. Louis, Mo.). The mononuclear cells were incubated with 1 mg of lysozyme at 37°C for 60 min and with 0.1% Triton X-100 and 2 mg of proteinase K at 65°C for a further 120 min. Phenol-chloroform-isoamylalcohol (25:24:1) extraction came next, and the DNA was precipitated by the addition of absolute ethanol.

PCR. PCR was carried out in three types of experiments. First, DNAs isolated from *Salmonella* spp. and other organisms were amplified to test the specificity of the PCR products. Second, the minimum detectable level by PCR was established by amplification of the serially diluted DNA from *S. typhi* ATCC 19430. To evaluate the influence of DNA from normally present leukocytes in the blood on the sensitivity of PCR, a known amount of DNA (2 µg) from mononuclear cells was added to serially diluted DNA from *S. typhi*. Finally, PCR was performed with DNAs isolated from the blood of 12 actual typhoid patients, 10 patients with other febrile diseases, and 4 patients with suspected typhoid fever.

The reaction mixture for the first round of PCR contained 2 µg of extracted DNA, 25 pmol (each) of ST 1 and ST 2, 200 µM (each) all four deoxyribonucleoside triphosphates, 0.625 U of *Taq* DNA polymerase (Boehringer Mannheim, Mannheim, Germany), and the standard PCR buffer (100 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl, 0.1% gelatin [pH 8.3]) in a final volume of 25 µl. Amplification in an automated DNA thermal cycler (Hybaid; Teddington, Middlesex, United Kingdom) consisted of 40 cycles at 94°C for 1 min (denaturation), 57°C for 1 min and 15 s (annealing), and 72°C for 3 min (polymerization). After the reaction, 5 µl of the amplified products of the first PCR was transferred to a second reaction mixture (20 µl) containing 25 pmol (each) of ST 3 and ST 4 for the nested PCR. The nested PCR was performed for 40 cycles at 94°C for 1 min, 68°C for 1 min and 15 s, and 72°C for 3 min.

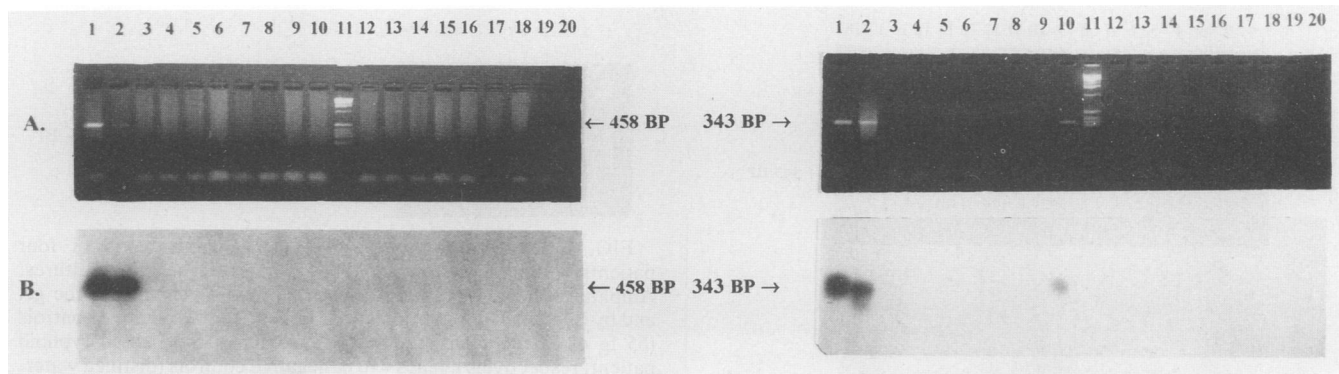


FIG. 1. Specificity of the PCR for detection of the flagellin gene of *S. typhi*. (Left) Amplification products of 458 bp from the single round of PCR were analyzed by electrophoresis through a 1.5% agarose gel (A) and by Southern blot hybridization with a ^{32}P -labeled internal probe (B). (Right) Amplification products of 343 bp from the nested PCR were analyzed by the same methods. Lanes 1 and 2, DNA isolated from *S. typhi* (ATCC and Asan Medical Center isolate); lanes 3 to 10, DNA isolated from other *Salmonella* strains; lanes 12 to 20, DNA isolated from other organisms; lanes 11, molecular weight marker (1-kb ladder from Bethesda Research Laboratories). The amount of template DNA was 4 ng, corresponding to 10^6 organisms. With the first round of PCR, amplification products were seen from the extracts of two *S. typhi* isolates, whereas with the nested PCR, *S. muenchen* (lanes 10 in right panels) as well as *S. typhi* was detected.

Detection of PCR products. The DNA fragments of the flagellin gene of *S. typhi* amplified by the PCR were identified by two different methods. In the agarose gel electrophoresis, 10 μl of the amplified products from both rounds of the PCR was electrophoresed on a 1.5% agarose gel for 60 min at a constant 80 V, with TBE buffer (90 mM Tris-borate, 2 mM EDTA). Molecular size markers (1-kb DNA ladder, Bethesda Research Laboratories) were run concurrently. The gels stained with ethidium bromide were examined under UV illumination for the presence of a 458- or 343-bp band. In Southern blot hybridization, a ^{32}P -labeled 40-base probe DNA located within the amplification product from the PCR was used. Capillary transfer of DNA from agarose gels to a GeneScreen Plus membrane (NEN, Boston, Mass.) was performed according to the manufacturer's instructions. Hybridization of oligonucleotide probe (3.0×10^8 cpm/ μg) was carried out in $6\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)– $5\times$ Denhardt's solution–0.5% SDS–100 μg of salmon sperm DNA per ml at 61°C for 16 h. After hybridization, membranes were washed in $6\times$ SSC, $5\times$ SSC, and $4\times$ SSC, consecutively, for 5 min each. The membranes were autoradiographed for 2 h by using X-Omat AR film (Eastman Kodak, Rochester, N.Y.) and intensifying screens at 80°C .

RESULTS

Specificity of the PCR. To verify that primers used in this study were specific for *S. typhi*, the nested PCR was carried out with DNAs from 10 *Salmonella* spp. and 9 other organisms. With the single round of PCR with ST 1 and ST 2, amplification products of the expected size (458 bp) were seen only from the extracts of two *S. typhi* strains but not from the extracts of other organisms both on the agarose gel electrophoresis and Southern blot hybridization (Fig. 1). With the nested PCR, however, amplification products of 343 bp were detected from the extracts of two *S. typhi* strains and *Salmonella muenchen* but not from the extracts of other organisms when analyzed on an agarose gel (Fig. 1). The pattern of hybridization obtained with a ^{32}P -labeled oligonucleotide was consistent with that of the agarose gel electrophoresis. The amount of template DNA from various bacteria was 4 ng (corresponding to 10^6 organisms).

Sensitivity of the PCR. To know the minimum detectable level, the nested PCR was carried out with DNA mixtures containing serially diluted DNA from *S. typhi* and mononuclear cell DNA. With the single round of PCR in the presence of 2 μg of mononuclear cell DNA, only 4 ng of *S. typhi* DNA (corresponding to 10^6 organisms) produced an amplification product of 458 bp on the gel (Fig. 2). The decreased sensitivity was believed to be an effect of nonspecific amplification, as would be expected. With the second round of PCR, however, 40 fg of target DNA (corresponding

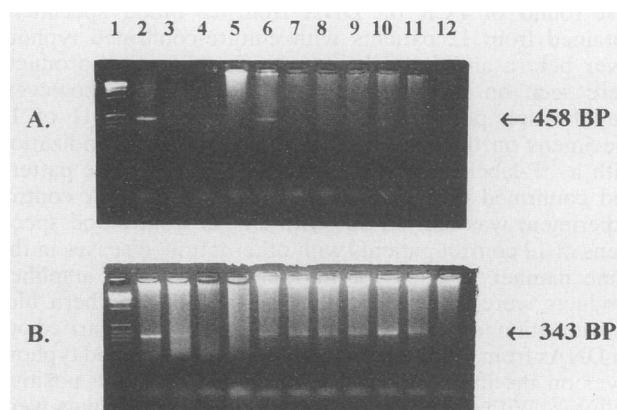


FIG. 2. Sensitivity of the PCR with serially diluted DNAs from *S. typhi* to which DNA from mononuclear cells was added. (A) With the first round of PCR, only 4 ng of *S. typhi* DNA (corresponding to 10^6 organisms) produced an amplification product of 458 bp on the gel because of nonspecific amplification. (B) The sensitivity of detection markedly increased after the nested PCR, which detected 40 fg of target DNA (corresponding to 10 organisms) on the gel. Lanes 2 and 3, positive controls (*S. typhi* DNA, 4 ng, and 4 fg without leukocyte DNA, respectively); lanes 4 and 5, negative controls (distilled water and leukocyte DNA); lane 6, *S. typhi* DNA, 4 ng; lane 7, *S. typhi* DNA, 400 pg; lane 8, *S. typhi* DNA, 40 pg; lane 9, *S. typhi* DNA, 4 pg; lane 10, *S. typhi* DNA, 400 fg; lane 11, *S. typhi* DNA, 40 fg; lane 12, *S. typhi* DNA, 4 fg; lane 1, molecular weight marker (1-kb ladder). *S. typhi* DNA in lanes 6 to 12 was mixed with 2 μg of mononuclear cell DNA.

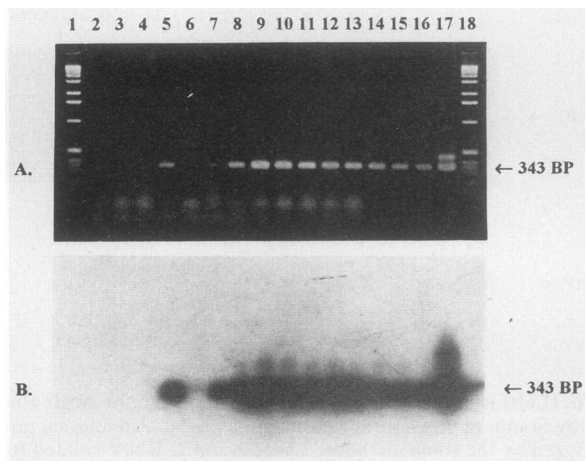


FIG. 3. Amplification of *S. typhi* DNA in the blood specimens from 12 patients with typhoid fever. Amplification products from the nested PCR were analyzed by gel electrophoresis (A) and by Southern blot hybridization (B). Amplification products were seen in 11 of 12 specimens on the gel and autoradiograph after the nested PCR (lanes 5 and 7 to 16). No amplification products were seen with the single round of PCR (not shown). Lanes 2 to 4, negative controls (distilled water, leukocyte DNA, and DNA from blood of a patient with *E. coli* bacteremia, respectively); lanes 5 to 16, DNAs isolated from blood of typhoid patients; lane 17, positive control (*S. typhi* DNA, 65 fg); lanes 1 and 18, molecular weight markers (1-kb ladder).

to 10 organisms) produced a visible amplification product of 343 bp on the gel (Fig. 2). These results suggested that the nested PCR was necessary to detect the small numbers of organisms in the actual blood specimens which normally contain leukocyte DNA in excess amounts.

PCR with DNA from blood specimens of patients. With the first round of PCR on DNA from 12 blood specimens obtained from 12 patients with culture-confirmed typhoid fever before antibiotic therapy, no amplification products were seen on the gel. After the nested PCR, however, amplification products of 343 bp were seen in 11 of 12 specimens on the gel (Fig. 3). Southern blot hybridization with a ^{32}P -labeled internal probe showed the same pattern and confirmed the agarose gel results (Fig. 3). A control experiment was carried out with DNAs from blood specimens of 10 control patients with other febrile diseases in the same manner as used for the nested PCR. No amplified products were observed on the gel and by Southern blot hybridization (data not shown). Finally, PCR was carried out on DNAs from blood of four patients with suspected typhoid fever on the basis of clinical features but without positive cultures. With the nested PCR, amplification products were seen in all four cases on the gel (Fig. 4).

DISCUSSION

The flagellar antigen of *S. typhi* (H1-d) is encoded by a 1,530-bp DNA sequence (4, 13). Although flagellar antigen is not a structure specific to *Salmonella* species and d antigen is also present in many *Salmonella* species other than *S. typhi* (1, 2), the flagellin gene of *S. typhi* has unique nucleotide sequences in the hypervariable region of the gene (4). The nucleotide sequences and predicted amino acid sequences of region VI (corresponding to nucleotide 969 to 1077) of the H1-d flagellin gene of *S. typhi* are different from those of *S. muenchen*, which has also the H1-d gene and

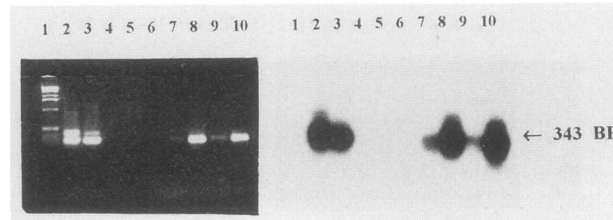


FIG. 4. Amplification of *S. typhi* DNA from blood of four patients with suspected typhoid fever but with negative cultures. Amplification products were seen in all four specimens on the gel and by Southern blot hybridization. Lanes 2 and 3, positive controls (65 fg of *S. typhi* DNA and DNA from blood of actual typhoid patient, respectively); lanes 4 to 6, negative controls (distilled water, leukocyte DNA, and DNA from blood of patient with *E. coli* bacteremia, respectively); lanes 7 to 10, DNA from blood of patients with suspected typhoid fever; lane 1, molecular weight marker (1-kb ladder).

nucleotide sequences highly homologous with *S. typhi* (4, 13). These findings suggested that the PCR test, based on the unique sequence in the flagellin gene of *S. typhi*, could be used to detect *S. typhi* specifically in the clinical specimens. In our study, PCR with ST 1 and ST 2 was highly specific in detecting *S. typhi* DNA, whereas the nested PCR with ST 3 and ST 4 was rather nonspecific, detecting amplification products both of *S. typhi* and *S. muenchen*. This result could be anticipated because the nucleotide sequences of ST 3 and ST 4 were highly homologous between *S. typhi* and *S. muenchen*. In view of the practical use of PCR, however, this finding is thought to be not critical because PCR technique can be used to reinforce the clinical diagnosis of typhoid fever in patients with suspected clinical features of typhoid fever, such as high fever, leukopenia, hepatosplenomegaly, and so on, but with negative cultures. *S. muenchen* is an uncommon etiologic agent of gastroenteritis which can be clearly differentiated from typhoid fever on the basis of clinical findings. Furthermore, the detection of *S. muenchen* in blood specimen would be impossible with this PCR technique even if it is present, because it is subjected to be amplified by only the second round of reaction (40 cycles) with ST 3 and ST 4, and not by the nested reaction, as for *S. typhi*. As shown in the sensitivity test for *S. typhi* DNA, the single-round reaction is insufficient to detect a small number of organisms in clinical specimens. The specificity of the PCR in clinical practice was confirmed by the results of PCR performed on DNA from blood samples of patients with other acute febrile diseases, which were consistently negative for *S. typhi* DNA.

Our strategy was to develop the PCR technique with which amplified fragments of flagellin gene of *S. typhi* in the blood could be directly detected on the gel without the use of Southern blot hybridization. For the practical use of the PCR as a diagnostic test, the gel electrophoresis should be sufficient to detect amplification products without the aid of hybridization methods which take at least 2 days with the use of radiolabeled probes. Because patients with typhoid fever generally have a very low level of bacteremia, the PCR should be sensitive enough to detect 1 to 10 organisms. As shown in a sensitivity test of the PCR performed on mixtures of DNA from mononuclear cells and *S. typhi* DNA, which could partly simulate the actual blood specimens, the nested PCR was necessary to detect approximately 10 organisms of *S. typhi* on the gel electrophoresis. The use of mononuclear cells instead of whole blood was to separate the bacteria

from erythrocyte debris or serum proteins which could interfere with the detection of *S. typhi* DNA in the blood.

The ultimate objective of our study was to use the PCR technique to detect *S. typhi* DNA in the blood of patients with typhoid fever. Using a DNA derived from isolated *Salmonella* strains, Frankel et al. amplified flagellin gene sequences specifically from *S. typhi* (4), but there have been no reports of a successful detection of *S. typhi* DNA in the clinical specimens by use of PCR. In our study, the PCR performed on DNA from the blood specimens successfully detected *S. typhi* DNA in 11 of 12 specimens on the gel, and Southern blot hybridization confirmed the gel results. Failure to detect *S. typhi* DNA in one case of actual typhoid fever (Fig. 3, lane 6) might be due to partial clotting of the stored blood sample which would result in a significant loss of extractable DNA from mononuclear cells. The PCR could detect *S. typhi* DNA in the normally prepared blood specimens.

The practical value of PCR in the clinical specimens is the detection of *S. typhi* DNA in the blood specimens from patients with suspected clinical findings but with negative cultures. The low level of bacteremia in typhoid patients can cause negative blood culture, particularly if the patient has been treated with antibiotics before cultures. This situation is relatively common in endemic areas such as Korea or Southeast Asia. Depending on the conventional diagnostic methods, these culture-negative cases cannot be confirmatively diagnosed as typhoid fever. Pretreatment with antibiotics also can cause some modifications of clinical features of typhoid fever and may lead to unnecessary workup for the cause of fever as well as improper treatment. In our study, PCR performed on DNA from the blood of four patients in this setting successfully detected amplification products in all blood specimens. On the basis of the results of the PCR, these patients were treated with ciprofloxacin (500 mg twice a day, orally) for 14 days with an excellent outcome. We minimized the possibility of false-positive results of the PCR by meticulous handling of the materials and the simultaneous application of multiple negative controls. This finding suggests that the PCR can reinforce the clinical diagnosis of typhoid fever in culture-negative cases and can avoid other unnecessary workup.

By using two pairs of primers evaluated in this study, amplification of the flagellin gene of *S. typhi* identified the presence of the organism in the blood of patients. The nested PCR resulted in amplified fragments that were visible after agarose gel electrophoresis, which can preclude the use of Southern blot hybridization. The whole procedure to identify *S. typhi* DNA in the blood by agarose gel electrophoresis took only 16 h, demonstrating the PCR to be a simple, specific, and rapid method for the early diagnosis of typhoid fever.

ACKNOWLEDGMENTS

This work was supported by research grant 1991 from the Asan Institute for Life Sciences.

We thank Y. Kim and J. S. Lee for help with revising the manuscript.

REFERENCES

1. Edelman, R., and M. M. Levine. 1986. Summary of an international workshop on typhoid fever. *Rev. Infect. Dis.* 8:329-349.
2. Ewing, W. H. 1986. Antigenic scheme for *Salmonella*, p. 247-318. In W. H. Ewing (ed.), *Identification of Enterobacteriaceae*. Elsevier, New York.
3. Frankel, G., S. M. C. Newton, G. K. Schoolnik, and B. A. D. Stocker. 1989. Intragenic recombination in a flagellin gene: characterization of the H1-j gene of *Salmonella typhi*. *EMBO J.* 8:3149-3152.
4. Frankel, G., S. M. C. Newton, G. K. Schoolnik, and B. A. D. Stocker. 1989. Unique sequences in region VI of the flagellin gene of *Salmonella typhi*. *Mol. Microbiol.* 3:1379-1383.
5. Gilman, R. H., M. Termini, M. M. Levine, P. Hernandez-Mendoza, and R. B. Hornick. 1975. Relative efficacy of blood, urine, rectal swab, bone marrow, and rose spot cultures for recovery of *Salmonella typhi* in typhoid fever. *Lancet* i:1211-1213.
6. Guerra-Caceras, J. G., E. Gotuzzo-Herencia, E. Crosby-Dagnino, J. Miro-Quesada, and C. Carillo-Parodi. 1979. Diagnostic value of bone marrow culture in typhoid fever. *Trans. R. Soc. Trop. Med. Hyg.* 73:680-683.
7. Hoffman, S. L., D. C. Edman, N. H. Punjabi, M. Lesmana, A. Cholid, S. Sundah, and J. Harahap. 1986. Bone marrow aspirate culture superior to streptokinase clot culture and 8 ml 1:10 blood-to-broth culture for diagnosis of typhoid fever. *Am. J. Trop. Med. Hyg.* 35:836-839.
8. Levine, M. M., O. Grados, R. H. Gilman, E. Woodward, R. Solis-Plaza, and W. Waldman. 1978. Diagnostic value of Widal test in areas endemic for typhoid fever. *Am. J. Trop. Med. Hyg.* 27:795-800.
9. Rubin, F. A., D. J. Kopecko, K. F. Noon, and L. S. Baron. 1985. Development of a DNA probe to detect *Salmonella typhi*. *J. Clin. Microbiol.* 22:600-605.
10. Rubin, F. A., D. J. Kopecko, R. B. Sack, P. Sudarmono, A. Yi, D. Maurta, R. Meza, M. A. Moehtar, D. C. Edman, and S. L. Hoffman. 1988. Evaluation of a DNA probe for identifying *Salmonella typhi* in Peruvian and Indonesian bacterial isolates. *J. Infect. Dis.* 157:1051-1053.
11. Rubin, F. A., P. D. McWhirter, D. Burr, N. H. Punjabi, E. Lane, S. Kumala, P. Sudarmono, S. P. Pulungsih, M. Lesmana, P. Tjaniadi, N. Sukri, and S. L. Hoffman. 1990. Rapid diagnosis of typhoid fever through identification of *Salmonella typhi* within 18 hours of specimen acquisition by culture of the mononuclear cell-platelet fraction of blood. *J. Clin. Microbiol.* 28:825-827.
12. Rubin, F. A., P. D. McWhirter, N. H. Punjabi, E. Lane, P. Sudarmono, S. P. Pulungsih, M. Lesmana, S. Kumala, D. J. Kopecko, and S. L. Hoffman. 1989. Use of a DNA probe to detect *Salmonella typhi* in the blood of patients with typhoid fever. *J. Clin. Microbiol.* 27:1112-1114.
13. Wei, L., and T. M. Joys. 1985. Covalent structure of three phase-1 flagellar filament proteins of *Salmonella*. *J. Mol. Biol.* 186:791-803.
14. Wicks, A. C. B., J. G. Cruickshank, and N. Musewe. 1974. Observations on the diagnosis of typhoid fever in an endemic area. *S. Afr. Med. J.* 48:1368-1370.